A simple egg membrane model for understanding diffusion characteristics of nanoparticles and amino acids

Deepika Tari, Shalaka Haryan, Kaustubh Patankar, Vinod Jaiswal, Mahesh Samant, S. Sivakami and P. M. Dongre*

Department of Biophysics, University of Mumbai, Vidyanagari, Santacruz (E), Mumbai 400 098, India

This study reports the passive diffusion (*in vitro*) of silver nanoparticles (SNPs) and those of the amino acids tryptophan, phenylalanine, tyrosine across a biological membrane model. The experiments were carried out under physiological conditions at pH 7.4. Chicken egg shell outer membrane model was used to study the passive diffusion of the above materials. Passive diffusion was performed against and towards gravitation for 24 and 48 h. Fick's first law of diffusion was adopted for quantification of diffusion coefficient, permeability constant and diffusion rate. The egg shell membrane was characterized using scanning electron microscopy. The SNPs were synthesized by chemical degradation method and characterized by UV-visible spectroscopy and dynamic light scattering. An average size of nanoparticles obtained was 62 nm. The diffusion rates of amino acids were higher than those of SNPs. However, they were enhanced in their presence. Permeability coefficient and diffusion coefficient were higher for amino acids than SNPs. The possible mechanisms have been explained on the basis of molecular properties.

Keywords: Amino acids, chicken egg shell membrane, diffusion rate, permeability constant, silver nanoparticles.

NANOMATERIALS possess unique optical, thermal, magnetic and electrical properties. As a result, these materials are being used in biological and medical applications such as bioimaging[1], therapy, drug delivery[2], etc. Large surface-to-volume ratio of nanoparticles (NPs) allows this class of materials to serve as efficient carriers of biological molecules. The fundamental interaction of NPs with biological systems remains is not well understood.

Upon incorporation of NPs in body fluid, they interact with biological materials that dissolve in body fluids such as proteins, carbohydrates, lipids before interaction with cells and cell membranes. It is reported that NPs enter the cell by passive diffusion through plasma membrane[3,4]. The entry of NPs into cells depends on physico-chemical surface properties, charge[5] and shape[6]. Uptake of different NPs by different cell lines can vary significantly in their kinetics[7]. The internalization of NPs due to contact with plasma membrane is a concentration-dependent process. The mechanisms of internalization of NPs at molecular and cellular level are yet to be understood.

Kim *et al.*[8] studied accumulation of NPs in human lung carcinoma cells (A549) and observed that NPs accumulate in lysosomes as observed by fluorescent technique. Jiang *et al.*[9] studied the uptake of polystyrene NPs by mesenchymal stem cell using spinning disc confocal optical microscope combined with quantitative image analysis. It is also reported that NPs of diameter 10 nm accumulate on the cell membrane prior to internalization[10]. However, ten-fold larger polymeric NPs were not observed to adsorb on the cell membrane. Some of the *in vitro* experiments showed cellular uptake of NPs exposure cell at the bottom of cellular plate. Cho *et al.*[11] studied the effect of uptake of gold NPs by human breast cancer cells (SK-BR-3, ATCC HTB-30). NPs may enter the cell through passive diffusion mechanisms, but details of such processes remain unsolved. Some of the efforts for understanding passive diffusion of NPs used RBC cell model[3,4].

Diffusion is an important phenomenon that occurs in living systems for carrying out various biological activities. This has been quantitatively described using mathematical models and proved experimentally. The outcome of these studies has been used for designing experiments *in vivo* and *in vitro*[12,13]. The simplest mechanism by which molecules can move across the membrane is passive diffusion. During passive diffusion, a molecule simply crosses the membrane and enters the aqueous solution at the other side of the membrane. In the case of plasma membrane, the molecule dissolves in the phospholipid bilayer and diffuses across it. No membrane proteins are involved and the direction of the transport is determined simply by relative concentration of the molecule inside and outside the cell. The net flow is always down their concentration gradient from the compartment with high concentration to one with lower concentration of the molecule. It is a non-selective process, with only small, relatively hydrophobic molecules being able to diffuse across the membrane.

Diffusion also takes place through protein channels which form an open pore in membrane, allowing small molecules of appropriate size and charge to pass freely through the membrane. One group of channel proteins is porins which permit free passage of molecules and ions through outer membrane of bacteria, and the other group consists of channel proteins that permit passage of molecules between connected cells. These mass transport processes, well characterized by the German physiologist Adolf Fick, is known as Fick's First Law. The transport theories have been described in many text books and research papers[12].

Different membranes, natural and synthetic, were employed to study the diffusion process. Synthetic membranes have been used for drug diffusion study, for example polydimethylsiloxane (PDMS) which serves as a

*For correspondence. (e-mail: drpmdongre@yahoo.co.in)

membrane model for drug diffusion[14]. Polyvinylidenedifluoride (PVDF) membrane was used to study the diffusion of non-steroid anti-inflammatory drug (NSAID), acetylsalicylic acid[15]. Petro *et al.*[16] tested synthetic cellulose acetate membrane model for anti-inflammatory drugs. Diffusion of hydrocortisone (steroid hormone) was studied using synthetic nylon membrane[17]. Some natural membranes like septate giant axon of earthworm, skin models have been employed for diffusion study[18].

The present work aims to test if silver nanoparticles (SNPs) and amino acids diffuse through the membrane. The chicken egg shell membrane model has been used to perform passive diffusion of SNPs and amino acids. Chicken egg consists of the inner and outer membrane with calcified shell. Chief function of the membrane is to prevent penetration of bacteria. Shell membrane is essential for the formation of egg shell. The egg shell and shell membrane contain protein as a major constituent with small molecules like carbohydrates and lipids[19]. This membrane has been characterized with the help of X-ray scattering and scanning electron microscopy (SEM)[20]. This study throws light on some biophysical aspects of passive diffusion of SNPs and amino acids.

Silver nitrate (qualigens) and trisodium citrate (SD, fine) were used to make SNPs in phosphate buffer of pH 7.4 (1 M) as solvent. All the chemicals were of analytical reagent grade and double-distilled water was used throughout. Outer shell membrane of eggs was used for the present study.

Silver nanoparticles were synthesized by chemical reduction method. The synthesis procedure was a slightly modified form of Lee and Meisel[21]. One hundred ml of silver nitrate (10^{-3} mol l^{-1}) was heated on a hot plate-cum-magnetic stirrer to 353 K; 1% trisodium citrate (50 ml) was added drop-wise till the colourless solution turned yellow. The colour change indicates the formation of SNP.

The number of silver atoms (N) in each SNP is 31 d^3 where d is the size of the nanoparticle in nm. Concentration of the SNP solution is calculated using the equation[22]

$$C = \frac{N_{\text{Total}}}{NVN_{\text{A}}}, \tag{1}$$

where N_{Total} is the total number of silver atoms added to the reaction solution, N the number of silver atoms present in each nanoparticle, V the volume of the reaction solution in litres and N_{A} is the Avogadro number.

The colloidal silver nanoparticle solution was characterized using UV-visible (Implen nanophotometer). UV-visible absorption spectrum was recorded in the wavelength range of 200–600 nm using a one cm path length quartz cuvette. Dynamic light scattering (DLS) measurements were performed on Microtrac Inc particle size analyser.

The aromatic amino acids (tryptophan, tyrosine and phenylalanine) were estimated using standard calibration graph.

A fresh chicken egg was carefully broken and its outer membrane was removed with necessary precautions without tearing/damaging. The membrane was kept in a petri dish with saline till further study. Then the membrane was transferred and fixed on the mouth of a 50 ml conical flask with a diameter of 1.7 cm. The upper flask contained SNP and the lower contained normal saline. The flux of silver nanoparticles/amino acids from lower flask was measured after 24 and 48 h. Scanning electron microscopic images of egg shell membranes were taken before, after and during experimentation (experiments were terminated at the middle stage and SEM performed).

Fick's first law of diffusion under steady state was adopted to evaluate the diffusion rate (J), diffusion coefficient (D) and change in concentration with thickness of membrane ($\mathrm{d}c/\mathrm{d}x$), by eq. (2)

$$J(x,t) = -\frac{Ddc(x,t)}{d(x)}. \tag{2}$$

Diffusion coefficient of solute was calculated by eq. (3)

$$D = \frac{KT}{6\pi r\eta}, \tag{3}$$

where K is the Boltzman constant (1.38×10^{-23} m^2 kg s^{-2} K^{-1}), T the temperature, r the radius of the molecules and η is the viscosity of the solution. Viscosity was estimated using Ostwald viscometer.

The permeability coefficient (P) was estimated according to Fick's law of diffusion based on the steady state flux and the concentration of the donor compartment using eq. (4)

$$P = \frac{J}{C_{\text{donor}}}, \tag{4}$$

viscosity of the solvent was determined of each solution, i.e. SNPs and amino acids using Ostwald viscometer and calculated using the eq. (5)

$$\eta_2 = \frac{\eta_1 \times \rho_1 \times t_2}{\rho_2 \times t_1}, \tag{5}$$

where η_1 is the viscosity, ρ_1 the density of the distilled water, t_1 the time taken by the distilled water to flow between the marks A and B on the viscometer. ρ_2 is the density of the test sample and t_2 is time taken by the test sample to flow between the marks A and B on the viscometer.

In the UV-visible absorption spectra, the peak at 420 nm indicated the formation of SNPs (Figure 1). Results of dynamic light scattering reveal the average size of SNPs to be 62 nm. The concentration of SNP was calculated to be 0.615×10^{-9} mol l^{-1}.

Egg shell membranes were examined under SEM at different magnifications (Figures 2 and 3). SEM images were taken before, after and middle positions during the experiment. The images revealed continuous sheet of fibres crossing each other in the limiting membrane. At higher magnification, layers of fibres were clearly distinguished. Various sizes of pore/holes were observed ranging from 2.28 to 5.62 µm due to crossing of the fibres. The presence of holes in shell membrane was noted by Simon[23]. However, the functional/physiological significance has not yet been reported. Tan *et al.*[24] also studied the chicken egg shell membrane using SEM. These structures were made up of organic and collagen protein fibres lying parallel to egg surface[25]. We believe that SNPs and amino acids might be passing through these crossed structures created by the fibres.

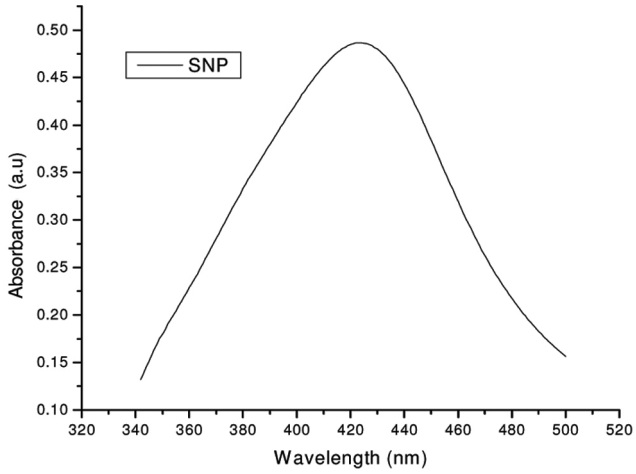

Figure 1. UV-visible absorption spectra of silver nanoparticles showing the peak at 420 nm.

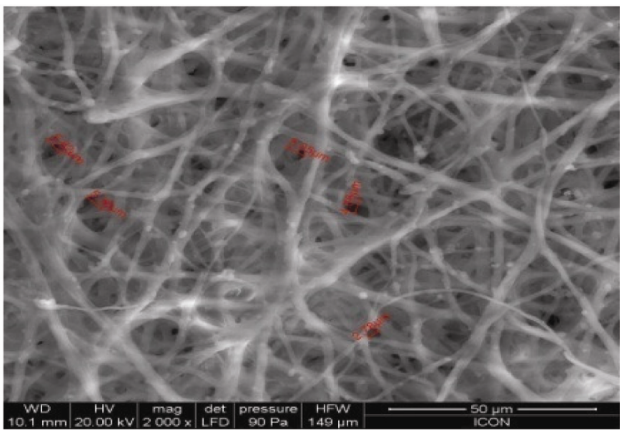

Figure 2. SEM image of fresh sample of egg shell membrane.

A schematic representation of experimental set-up is shown in Figure 4. The molecular mass transport in solutions/across biological membrane is usually determined in fluxes. The amount of flux is concerned with number of molecules and cross-section area of membrane and over specific periods of time. Fick's first law steady state is most appropriate for analysis of the flux (eq. (2)). In the present study, the amount of SNP and amino acid diffused to receiver compartment was determined at 24 and 48 h against as well as towards gravitation (Table 1). The experiment clearly indicates the amount of SNP and amino acids that have crossed membrane barrier and entered the receiver compartment. It was observed that the rate of diffusion (J) of SNP and amino acids was higher towards gravitation than against gravitation (Table 1). The diffusion is due to gravitation force, hydrodynamic and concentration gradient/hydrodynamic force imparting on the particles. However, the rate of change of diffusion is associated with diffusion coefficient (D) of the particles, which depends on factors related to size, shape and type of medium, i.e. viscous/nonviscous. The diffusion coefficient (D) of SNP and amino acids was calculated according to eq. (3). Diffusion coefficient of SNP was noticed to be lower than that of free amino acids which could be due to the size difference between SNP (62 nm) and amino acids[26] Phe-3.56 Å, Tyr-3.58 Å, Trp-3.78 Å. Thus diffusion coefficient is seen to decrease with increasing particle size.

In the current experiment, the fluxes were measured by simply taking sample from receiver compartment at given time intervals (24 and 48 h). The concentration gradient was observed to increase linearly in the receiver compartment. The permeability constant was determined using eq. (4) which showed that fluxes were proportional to concentration gradient. The permeability constant of SNP was lower than those of amino acids in experimental set-up towards gravity as compared to against gravity (Table 1).

We further tried to study whether the diffusion rates of amino acids increase or not in the presence of SNP. Interestingly it was found that the diffusion rate of amino acids increased in their presence (Table 1). Here the possible reason is that due to positive charges on SNP the amino acids could adsorb/bind to them causing an increase in diffusion rate. We have extensively studied the binding of proteins to SNP surface[22,27]. Our unpublished results indicate that amino acids adsorb on SNP as seen by Raman spectroscopic studies.

This study confirms that nanoparticles and amino acids crossed the outer egg shell membrane both against as well as towards gravity. The passive diffusion of SNPs and amino acids (Try, Tyr, Phe) has been studied and diffusion rate, permeability constant and diffusion coefficient have been quantitatively determined. The diffusion rates of amino acids in the presence of SNP were higher than those of the individual amino acids. Scanning electron

Table 1. Diffusion rate (J), permeability coefficient (P) and diffusion coefficient (D) of SNP and amino acids

Particles	Time (h)	Towards gravity			Against gravity		
		D (m^2 s^{-1})	J (mol m^{-2} s^{-1})	P (ms^{-1})	D (m^2 s^{-1})	J (mol m^{-2} s^{-1})	P (ms^{-1})
SNP	24	7.411×10^{-13}	7.940×10^{-16}	12.91×10^{-10}	7.411×10^{-13}	7.412×10^{-16}	12.56×10^{-10}
	48		11.54×10^{-16}	18.76×10^{-10}		10.004×10^{-16}	16.267×10^{-10}
Trp	24	4.476×10^{-10}	1.316×10^{-6}	5.371×10^{-6}	4.476×10^{-10}	1.190×10^{-6}	4.859×10^{-6}
	48		1.9118×10^{-6}	7.803×10^{-6}		1.864×10^{-6}	7.610×10^{-6}
Trp + SNP	24	4.58×10^{-10}	2.821×10^{-6}	11.51×10^{-6}	4.58×10^{-10}	1.635×10^{-6}	6.676×10^{-6}
	48		4.361×10^{-6}	17.80×10^{-6}		2.276×10^{-6}	9.293×10^{-6}
Tyr	24	4.917×10^{-10}	1.552×10^{-6}	5.615×10^{-6}	4.917×10^{-10}	1.416×10^{-6}	5.126×10^{-6}
	48		1.7652×10^{-6}	6.390×10^{-6}		1.534×10^{-6}	5.553×10^{-6}
Tyr + SNP	24	5.123×10^{-10}	1.859×10^{-6}	6.730×10^{-6}	5.123×10^{-10}	1.738×10^{-6}	6.292×10^{-6}
	48		2.263×10^{-6}	8.193×10^{-6}		1.798×10^{-6}	6.509×10^{-6}
Phe	24	5.24×10^{-10}	1.269×10^{-6}	4.190×10^{-6}	5.24×10^{-10}	1.156×10^{-6}	3.816×10^{-6}
	48		2.313×10^{-6}	7.633×10^{-6}		2.018×10^{-6}	6.660×10^{-6}
Phe + SNP	24	5.331×10^{-10}	2.630×10^{-6}	8.681×10^{-6}	5.331×10^{-10}	1.961×10^{-6}	6.471×10^{-6}
	48		4.015×10^{-6}	13.25×10^{-6}		3.460×10^{-6}	11.419×10^{-6}

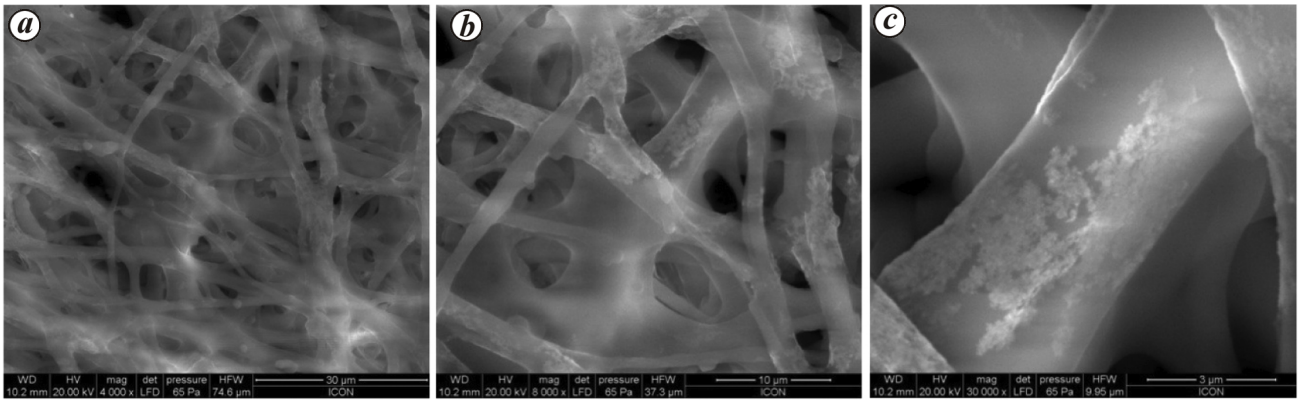

Figure 3 *a*–*c*. SEM images at different magnifications (during experimentation).

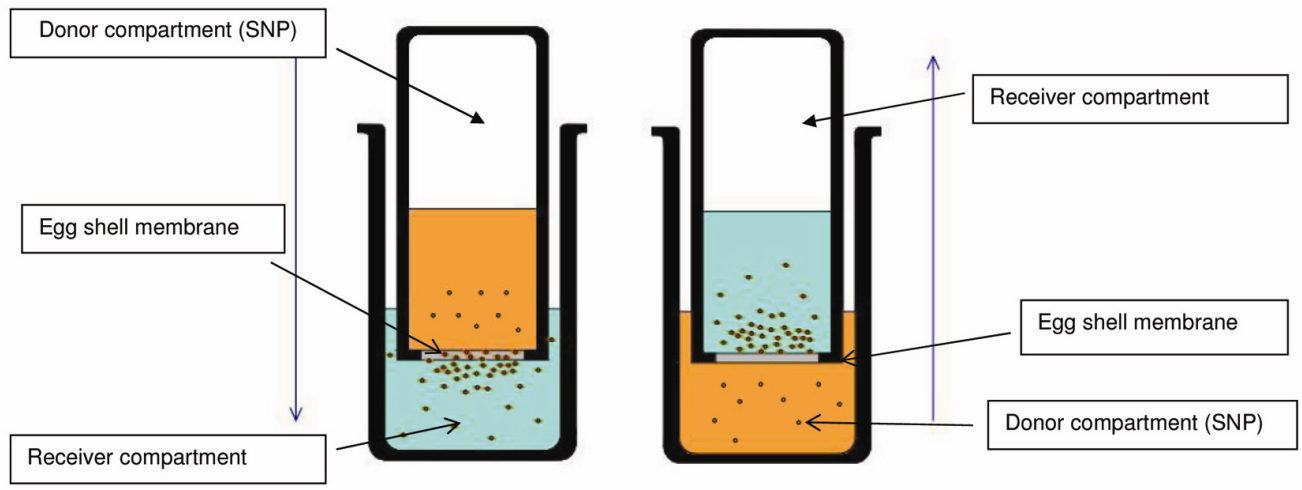

Figure 4. Schematic experimental set up of diffusion of particles against and towards gravity (↑↓).

microscopy reveals that pores are formed due to crossing of protein structures with various dimensions. The molecules could be diffusing through these membrane pores.

Since the major application of diffusion is in pharmaceutical, food and beverage industries and in clinical application such as MRI/bio imaging, hemodialysis, this model could be useful for screening/characterization of biological materials *in vitro*. Based on the quantification of diffusion parameters of particles, it is possible to predict their possible behaviour *in vivo*. However, various interfering factors need to be understood. This report describes a simple and inexpensive experiment which throws light on biophysics of membrane model. Further, this could be a good experimental model for learning and designing experiments in teaching and research lab. We believe that this to be the first *in vitro* experimental report of biophysical study of diffusion characteristics of silver nanoparticles and amino acids.

1. Korkusuz, H. *et al*., Transferrin-coated gadolinium nanoparticles as MRI contrast agent. *Mol. Imaging Biol*., 2013, **15**(2), 148–154.
2. Asadishad, B., Vossoughi, M. and Alemzadeh. Folate-receptor-targeted delivery of doxorubicin using polyethylene glycol-functionalized gold nanoparticles. *Ind. Eng. Chem. Res*., 2010; **49**(4), 1958–1963.
3. Wang, T., Bai, J., Jiang, X. and Nienhus, G. V., Cellular uptake of nanoparticles by membrane penetration: a study of combining confocal microscopy with FTIR spectrochemistry. *ACS Nano*, 2012, **6**, 1251–1259.
4. Rothen-Rutishauser, B. M., Schurch, S., Haenni, B., Kapp, N. and Gehr, P., Interaction of fine particles and nanoparticles with red blood cells visualized with advanced microscopic techniques. *Environ. Sci. Technol*., 2006, **40**, 4353–4359.
5. Labhshetwar, V., Song, C., Humphery, W., Shebuski, R. and Levy, R. J., Arterial uptake of biodegradable nanoparticles: effect of surface modification. *J. Pharm. Sci*., 1998, **87**, 1229–1234.
6. Chithrani, B. D., Ghazani, A. A. and Chan, W. C. W., Determining the size and shape dependence of gold nanoparticles uptake into mammalian cells. *Nano Lett*., 2006, **6**, 662–668.
7. Li, Y., Yue, T., Yang, K. and Zhang, X., Molecular modeling of the relationship between nanoparticle shape anisotropy and endocytosis kinetics. *Biomaterials*, 2012, **33**, 4965–4973.
8. Kim, J. A., Aberg, C., Salvati, A. and Dawson, K. A., Role of cell cycle on the cellular uptake and dilution of nanoparticles in cell population. *Nat. Nanotechnol*., 2012, **7**, 62–68.
9. Jiang, X., Musyanovych, A., Rocker, C., Landfester, K., Mailander, V. and Nienhaus, G. U., Specific effects of surface carboxyl groups on anionic polysterene particle in their interaction with mesenchymal stem cell. *Nanoscale*, 2011, **3**, 2028–2035.
10. Jiang, X., Weise, S., Hafner, M., Rocker, C., Zhang, F., Parkar, N. J. and Nienhaus, G. U., Quantitative analysis of the protein corona on FePt nanoparticles formed by transferring binding. *J. R. Soc. Interface*, 2010, **7**, S5–S13.
11. Cho, E. C., Zhang, Q. and Xia, Y., The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles. *Nat. Nanotechnol*., 2011, **6**, 385–391.
12. Schultz, S. G., *Basic Principle of Membrane Transport*, Cambridge University Press, Cambridge, 1980.
13. Steen-Knudsen, O., *Biological Membranes, Theory of Transport, Potentials and Electric Impulses*, Cambridge University Press, Cambridge, 2012.
14. Twist, J. N. and Zatz, J. L., Influence of solvents on paraben permeation through idealized skin model membrane. *J Sac. Cosmet. Chem*., 1986, **37**, 429–444.
15. Olivella, M., Debattisa, N. B. and Pappano, N. B., Salicyclic acid permeation: a comparative study with different vehicles and membranes. *Biocell*, 2006, **30**(2), 321–324.
16. Petro, E. *et al*., *In vitro* and *in vivo* evaluation of drug release from semisolid dosage form. *Pharmazie*, 2011, **66**, 936–941.
17. Christensen, J. M., Chang, M. C., Le, H. and Ehab Bendas. Hydrocortisone diffusion through synthetic membrane, mouse skin and Epiderm^{TM} cultured skin. *Arch. Drug Info*., 2011, **4**, 10–21.
18. Brink, P. R. and Ramanan, S. V., A model for diffusion of fluorescent probes in the septate giant axon of earthworm. *Biophys. J*., 1985, **48**, 299–309.
19. Burley, R. W. and Vedehra, D. V., The egg shell and shell membrane: properties and synthesis. In *Avian Egg, Chemistry and Biology*, John Wiley, New York, 1989, pp 25–64.
20. Lammie, D., Bain, M. M. and Wess, T. J., Microfocus X-ray scattering investigation of egg shell nanotexture. *J. Synchrot. Rad*., 2005, **12**, 721–726.
21. Lee, P. C. and Meisel, D., Adsorption of surface enhanced Raman of dyes on silver and gold sols. *J. Phys. Chem*., 1982, **86**, 3391–3395.
22. Jessy, M., Dongre, P. M. and Kothari, D. C., Study of interaction of silver nanoparticles with bovine serum albumin using fluorescence spectroscopy. *J. Fluor*., 2011, **21**; doi:101007/s10895-011-1922-3.
23. Simon, P. C. M., Ultrastructure of hen egg-shell and its physiological interpretation, PhD Thesis, Landbouwhoge School, Wageningen, Netherlands, Centre for Agricultural Publishing and Documentation, 1971.
24. Tan, C. K., Chen, T. W., Chen, H. L. and Ng, L. S., A scanning and transmission electron microscopic study of the membranes of chicken egg. *Histol. Histopathol*., 1992, **7**, 339–345.
25. Nys, Y. and Gautron, J., In *Bioactive Egg Compounds*, Ch. 15, Springer, Berlin, Heidelberg, 2007, pp. 99–102.
26. Zamyatinin, A. A., Protein volume solution. *Prog. Biophys. Mol. Biol*., 1972, **24**, 107–123.
27. Jessy, M., Sivakami, S. and Dongre, P. M., Bioactivity of albumins bound to silver nanoparticles. *Protein J*., 2014, **33**(3), 258–266.

Received 1 June 2015; revised accepted 7 November 2016

doi: 10.18520/cs/v112/i07/1574-1578